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American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*

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ABSTRACT

After more than a century of American Foulbrood (AFB) research, this fatal brood infection is still among the most deleterious bee diseases. Its etiological agent is the Gram-positive, spore-forming bacterium *Paenibacillus larvae*. Huge progress has been made, especially in the last 20 years, in the understanding of the disease and of the underlying host-pathogen interactions. This review will place these recent developments in the study of American Foulbrood and of *P. larvae* into the general context of AFB research.

1. Introduction

Animal pollination, i.e. pollination through insects, birds, bats, etc., is important for the sexual reproduction of many crops, fruit, and the majority of wild plants. Among the animal pollinators, insect pollinators, especially solitary and social bees, play the major role. In addition to their role in pollinating wild plants, managed honeybees (mainly *Apis mellifera*) are the most economically valuable pollinators of crop and fruit monocultures worldwide. Honeybees are cheap, versatile, and convenient, and often the only solution for farmers to ensure pollination when other pollinating species are lacking or scarce. Therefore, honeybees are among the most important productive livestock (Morse and Calderon, 2000). However, honeybees are attacked by numerous pathogens like viruses, bacteria, fungi and parasites which pose a significant threat to bee health. Due to the vital role honeybees play in the pollination of crops, fruit and wild flowers, factors affecting bee health also affect a sustainable and profitable agriculture as well as many non-agricultural ecosystems. Prior to the arrival of the parasitic mite, *Varroa destructor*, the economically most important diseases of honey bees worldwide were the bacterial brood diseases European Foulbrood (EFB) and American Foulbrood (AFB). AFB is still among the most deleterious bee diseases. It not only kills infected larvae but it is also potentially lethal to infected colonies. Under normal beekeeping conditions, AFB is highly contagious since spread of the disease is facilitated by exchanging hive and bee material between colonies, managing numerous hives in a confined area and the trading of queens, colonies (“package bees”) and honey. In many countries, AFB is a notifiable disease and measures are regulated by corresponding laws. Burning of colonies and contaminated hive material are widely considered the only workable control measure for diseased colonies whereas the shook swarm method (shaking the bees onto new comb foundation and destroying the infected comb) is sometimes applied to sanitize infected, although not yet clinically diseased colonies (Pernal et al., 2008; von der Ohe, 2003). Thus, AFB is a serious problem in apiculture and causes considerable economic loss to beekeepers all over the world.

More than a century ago, George F. White, who first isolated the etiological agent of AFB, wrote (White, 1906): “In order to combat a disease to the best advantage it is clear that its cause must be known, as well as the means by which the infection is transmitted and the environmental conditions which are favorable for the breaking out of an epidemic.” This review will summarize recent developments in the field of AFB research to see how far we have come to understand the causative agent of AFB and the pathogenesis of this bacterial infection within the last century and how close we are to combat the disease.

2. Pathogenesis of American Foulbrood

American Foulbrood (AFB), as its name suggests, only affects the larval stages of honeybees. The causative agent of AFB is the Gram-positive, spore-forming bacterium *Paenibacillus larvae* (Genersch et al., 2006). The extremely tenacious endospores are the only infectious form of this organism. The spores are infectious only for larvae; adult bees do not become infected upon ingestion of *P. larvae* spores (Hitchcock et al., 1979; Wilson, 1971). Larvae are most susceptible to infection during the early larval stages, i.e. 12–36 h after egg hatching. During this time window, the oral uptake of a dose of about ten spores or fewer via contaminated larval food is sufficient to successfully initiate a fatal infection (Bamrick and Rothenbuhler, 1961; Brodsgaard et al., 1998; Genersch et al., 2005; Woodrow, 1942; Woodrow and Holst, 1942). The

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following sequence of events in infected larvae could be demonstrated: Ingested spores pass through the foregut and germinate in the larval midgut around 12 h after ingestion (Bamrick, 1967; Yue et al., 2008). Historical studies suggested that vegetative *P. larvae* penetrate the midgut epithelium via phagocytosis immediately after germination and that the primary place of bacterial proliferation is the hemocoel (Bailey and Ball, 1991; Davidson, 1973). This was disproved recently using fluorescence *in situ* hybridization (FISH) and a *P. larvae* specific 16S rRNA probe (Yue et al., 2008). We now know, that vegetative bacteria colonize the midgut and massively proliferate there without visibly destroying the tissue integrity of the midgut epithelium (Yue et al., 2008). During this stage of infection, *P. larvae* obviously follows a commensal life style living from the food ingested by the larva. This is consistent with earlier findings that *P. larvae* contains active enzymes of the Emden-Meyerhoff-Parnas, pentose phosphate, and Entner-Doudoroff pathways involved in carbohydrate metabolism and that it is able to metabolize different sugars including glucose and fructose to support vegetative growth (Julian and Bulla, 1971; Neuendorf et al., 2004). In infected larvae, the peritrophic membrane helps to retain the bacterial mass in the midgut lumen although *P. larvae* is able to penetrate this protective layer (Davidson, 1970; Yue et al., 2008) and to attack the epithelium at a later stage of the infection when the larval gut is massively filled with these pathogenic bacteria. Breaching of the epithelium occurs via the paracellular route, i.e. the cells move through the paracellular space to enter the hemocoel where they also live (migrate and proliferate) in the paracellular space (Fig. 1). One characteristic of *P. larvae* is that it secretes highly active extracellular proteases during vegetative growth and infection (Dancer and Chantawannakul, 1997; Holst, 1946; Holst and Sturtevant, 1940; Hrabak and Martinek, 2007). It is tempting to speculate that some of these proteases are responsible for the disruption of the epithelial barrier integrity by degrading cell–cell and cell–matrix junctional structures thereby allowing *P. larvae* to invade the hemocoel. Proteases are further needed by *P. larvae* for the subsequent degradation of the larval remains to a brownish, semi-fluid, glue-like colloid (ropy stage). Both processes are vital for *P. larvae* since it must somehow escape the larval integument while still in the vegetative stage to ensure that after sporulation the spores are freely accessible for being distributed in the bee colony and swallowed by the next host. The ropy stage (Figs. 2–4) dries down to a hard scale (foulbrood scale) tightly adhering to the lower cell wall. These scales are highly infectious since they contain millions of spores which drive disease transmission within and between colonies (Bailey and Ball, 1991; Gregorc and Bowen, 1998; Lindström et al., 2008a; Sturtevant, 1932). The spores remain infectious for more than 35 years and withstand heat, cold, draught and humidity (Hasemann, 1961). It is the tenaciousness of the spores and the production of extremely high numbers of spores in diseased colonies that makes the effective control of AFB so difficult.

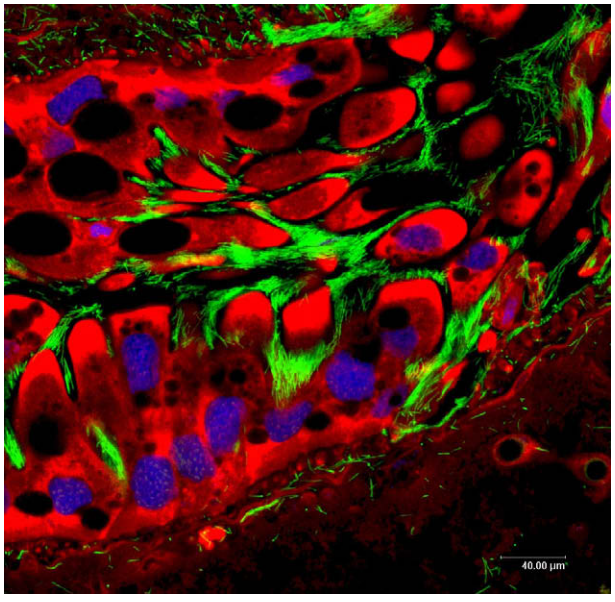

Fig. 1. Fluorescence *in situ* hybridization (FISH) analyzes of larvae infected with *P. larvae* ERIC I. *P. larvae* vegetative bacteria (green) were detected via a species-specific probe hybridizing to the 16S rRNA of *P. larvae*. Honeybee larval cells (red with blue nuclei) were visualized via a probe complementary to a unique target site on the 18S rRNA of many eukaryotes and nuclei were stained with DAPI (for experimental details see Yue et al. (2008)). Vegetative *P. larvae* can only be seen between larval cells. No bacteria could be demonstrated intracellularly.

3. Classification of *Paenibacillus larvae*

In the 18th century a honeybee brood disease was described which was characterized by a foul smell coming from the diseased colony and, hence, the name 'Foulbrood' was coined (Schirach, 1769). More than a century later, the first hint that 'Foulbrood' might actually comprise two different diseases can be found in the literature. According to Dzierzon (1882), 'Foulbrood' occurred with two different etiopathologies: 'Mild and curable' of unsealed brood (most probably what we now call European Foulbrood) and 'malignant and uncurable' of sealed brood (almost certainly American Foulbrood). In 1885, *Bacillus alvei* was isolated from diseased larvae and identified as causative agent of the disease at that time still simply known as 'Foulbrood' (Cheshire and Cheyne, 1885). In 1906, the American microbiologist White failed to isolate *B. alvei* from ropy mass and instead cultivated an unknown bacterium in pure culture. Based on the rod-shaped morphology and the ability to form endospores he classified the bacterium, that was consistently found in diseased and dead larvae, as *Bacillus larvae* (White, 1906). He was the one who also realized that there were two different foulbrood diseases caused by different pathogens developing different symptom complexes. On the one hand European Foulbrood caused by *Melissococcus plutonius* with the saprophytes *B. alvei* and/or *Enterococcus faecalis* as frequent secondary

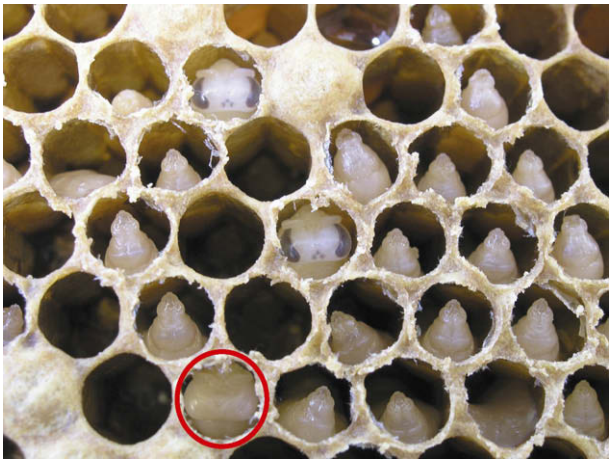

Fig. 2. Brood comb region originating from an experimentally infected mini colony. Uninfected larvae developed into dark-eyed pupae. Those infected larvae that were not removed by nurse bees died as engorged larvae and are about to be degraded by *P. larvae* into the characteristic ropy mass. One infected larva succeeded in undergoing metamorphosis and developed into a diseased pupa (red circle). Cells were carefully opened at day 13 after experimental infection of L1 larvae (around 12 h after egg hatching) to expose the cell content (for experimental details see Rauch et al. (2009)).

Fig. 3. Healthy pupa (left) and AFB diseased pupa (right) of the same age. The diseased pupa is the one seen in Fig. 2 (red circle). Diseased pupae are an extremely rare event, because infected larvae normally die as engorged larvae at the latest.

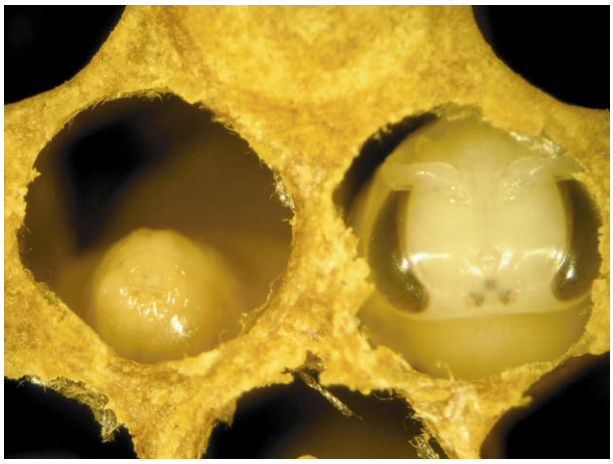

Fig. 4. Ropy mass from an infected larva (left) and a healthy dark-eyed pupa (right) of the same age. Both cells were carefully opened at day 13 after experimental infection of L1 larvae (around 12 h after egg hatch) to expose the cell content (for experimental details see Rauch et al. (2009)).

invaders (Bailey, 1956, 1957, 1983; Bailey et al., 1973); on the other hand American Foulbrood with *B. larvae* identified as the etiological agent (White, 1906).

In 1950, another bacterium was isolated from dead honeybee brood dried down to a powdery scale ('powdery scale disease') and classified as *Bacillus pulvifaciens* (Katznelson, 1950). Conflicting reports exist in the literature concerning the relation between *B. pulvifaciens* and the so-called 'powdery scale disease' of honeybee larvae (Hitchcock et al., 1979; Katznelson and Jamieson, 1951) and it took more than 50 years to finally answer the question about the pathogenicity and virulence of this bacterium (Genersch et al., 2006).

When comparative small-subunit rRNA (16S rRNA) sequence analysis was introduced into bacterial taxonomy it became evident that the genus *Bacillus* comprised five phyletic lines, rRNA group 1–5 bacilli (Ash et al., 1991), making an extensive taxonomic revision necessary. One of these lines, the rRNA group 3 bacilli proved to be sufficiently distinct to warrant reclassification in a new genus, *Paenibacillus* (Ash et al., 1993). Using a highly specific gene probe based on characteristic signatures within the 16S rRNA both *B. larvae* and *B. pulvifaciens* were assigned to the new genus *Paenibacillus* and hence, reclassified as *P. larvae* and *Paenibacillus pulvifaciens*, respectively (Ash et al., 1993). However, analysis of several type and reference strains of both species using a polyphasic approach revealed a high level of molecular similarity not justifying the classification into two species. In particular the rDNA restriction patterns and DNA-DNA-binding studies supported the reclassification of the two species into one species, *P. larvae* (Heyndrickx et al., 1996). At the infraspecific level, the species *P. larvae* was classified into two subspecies, *P. larvae* subsp. *larvae* and *Paenibacillus larvae* subsp. *pulvifaciens*, based on the phenotypic and genotypic differences as well as on the different pathologies described for the two former species (Heyndrickx et al., 1996).

However, studies on the characteristics of strains isolated from diseased colonies were contradictory to what was specified in the emended descriptions of the two subspecies. One example was the ability to produce an orange pigment solely ascribed to *P. l. pulvifaciens* (Heyndrickx et al., 1996) although orange pigmented colony variants had already been isolated from dead larvae originating from AFB diseased colonies (Drobnikova et al., 1994). The two subspecies were also described to differ in their ability to produce acid from salicin and mannitol (Heyndrickx et al., 1996) although the fermentation of salicin and mannitol had been reported to be a rather variable characteristic of field strains of *Paenibacillus larvae larvae* (Carpana et al., 1995; Dobbelaere et al., 2001). Finally, the 16S rRNA gene sequences proved to be identical for the two type strains of *P. l. larvae* and *P. l. pulvifaciens* (Kilwinski et al., 2004) and the resulting problems in the development of a PCR-test suitable for the differentiation between the pathogenic agent of American Foulbrood (*P. l. larvae*) and the presumably non-pathogenic close relative *P. l. pulvifaciens* (De Graaf et al., 2006) shed reasonable doubt on the correct classification of the species. Consequently, the most recent revision of this species led to the reclassification of the subspecies *P. l. larvae* and *P. l. pulvifaciens* as one species *P. larvae* without subspecies differentiation. Classical methods (colony morphology, electron microscopical analysis of spore morphology) and state-of-the-art molecular methods like pulsed-field gel electrophoresis (PFGE), protein profiling via SDS–PAGE analysis, and repetitive-element PCR (rep-PCR) (Versalovic et al., 1994) using enterobacterial repetitive intergenic consensus (ERIC) primers were applied not only to several type and reference strains of both subspecies but also to a collection of field strains isolated from AFB diseased colonies in Germany, Sweden, and Finland (Genersch et al., 2006). The results obtained confirmed and extended the high level of similarity and did not support the identity of the presumed *P. l. pulvifaciens* reference strain as a separate subspecies. Finally, exposure bioassays demonstrated that all strains irrespective of their affiliation to either subspecies were pathogenic for larvae (Tab. I). Infected larvae died showing symptoms of AFB and larval remains were degraded to the characteristic ropy mass. Hence, the strongest argument for the two different subspecies (Heyndrickx et al., 1996), i.e. the differences in the pathologies of *P. l. larvae* and *P. l. pulvifaciens* (Gilliam and Dunham, 1978; Katznelson, 1950; White, 1906), had been proven invalid resulting in reclassification of the former subspecies into one species without subspecies differentiation (Genersch et al., 2006). A summary of the characteristics of the four *P. larvae* ERIC genotypes is given in Table 1. Due to this rather frequent reclassification of the etiological agent of AFB, the pathogen can be found in the literature as *B. larvae* (White, 1906) having a close relative *B. pulvifaciens* (Katznelson, 1950), as two separate species *P. larvae* and *P. pulvifaciens* (Ash et al., 1993), as two subspecies *P. l. larvae* and *P. l. pulvifaciens* (Heyndrickx et al., 1996), and finally as single species *P. larvae* (Genersch et al., 2006). This has to be taken into account when searching for AFB related papers in the literature databases. For those who enter the field of AFB research subsequent to the most recent reclassification it should be noted that

Table 1
Characteristics of *P. larvae* genotypes ERIC I, II, III, and IV (Genersch et al., 2006).

Species Genersch et al. (2006)	*Paenibacillus larvae*			
Former subspecies Heyndrickx et al. (1996)	*P. l. larvae*		*P. l. pulvifaciens*	
ERIC genotypes	ERIC I	ERIC II	ERIC III	ERIC IV
Pathogenic, AFB symptoms	Yes	Yes	Yes	Yes
LT_{100}	~12 d	~7 d	~7 d	~7 d
Pest-like disease progression	Yes	Yes	Not yet observed	Not yet observed
Pigmented colony morphology	No	Yes	Yes	No
Hemolysis on CSA	No	No	Yes	(Yes)
Fermentation of mannitol	No	Yes	Yes	Yes
Fermentation of salicin	Yes	No	No	No

Notes: LT_{100}, time it takes the pathogen to kill all infected individuals (lethal time); CSA, Columbia sheep blood agar.

P. larvae strain designations like PLL and PLP (e.g. Antunez et al., 2007) are remnants of the former subspecies classification.

4. Epidemiology of Paenibacillus larvae

One focus of the last decade was the epidemiology of *P. larvae*. Outbreaks of AFB – or infectious diseases in general – often result from exposure to a common source of the pathogen. Hence, although at a given time several hosts are infected and these hosts may be spatially distributed the etiological agent in all these cases originally derived from a single cell. Hence, all pathogens isolated from the hosts involved in the outbreak are clonally related, i.e. they are members of the same species and share certain phenotypic and genotypic characteristics. Likewise, temporally distributed outbreaks may be related to a common source or to each other and, therefore, the etiological agents may again be clonally related. To follow the movements and the development of a pathogenic species in a host population epidemiological studies are conducted. In general, epidemiological studies investigate the temporal and spatial distribution of infectious diseases and attempt to determine the source of the infection, to reveal the relationship between different outbreaks, and to identify the factors influencing outbreaks.

Epidemiological studies are based on subtyping of the isolated strains. The method chosen for subtyping a specific pathogenic species must meet several criteria: (i) all strains of the species must be typeable with the chosen method, (ii) the method must have high differentiation power and (iii) must yield reproducible results within a laboratory (intralaboratory reproducibility) as well as between laboratories (interlaboratory reproducibility). Reproducibility is especially important for the construction of reliable databases from different laboratories. Phenotype based typing methods have several shortcomings especially with respect to applicability to all members of a species. Therefore, typing methods based on the microbial genotype or a particular DNA sequence have been established minimizing problems with typeability and reproducibility. Suitable molecular typing methods are for instance PFGE of whole chromosomal DNA (Schwartz and Cantor, 1984; Tenover et al., 1995), restriction fragment length polymorphism (RFLP) within defined genetic regions (Kühn et al., 1995), random amplified polymorphic DNA (RAPD) assays (Williams et al., 1990), amplified fragment length polymorphism (AFLP) based on the selective amplification of a subset of DNA fragments generated by restriction enzyme digestion (Vos et al., 1995), rep-PCR based on PCR amplification of repetitive elements present within bacterial genomes (Versalovic et al., 1994).

For subtyping of *P. larvae* isolates, several methods have been applied over the years. Restriction endonuclease fragment patterns (REFP) and RFLP did not become accepted (Alippi et al., 2002; Djordjevic et al., 1994). Instead, several studies describe the successful use of rep-PCR for subtyping of *P. larvae*. There are three main sets of repetitive elements used for bacterial subtyping: repetitive extragenic palindromic (REP) elements (Higgins et al., 1982; Stern et al., 1984), enterobacterial repetitive intergenic consensus (ERIC) sequences (Hulton et al., 1991; Sharples and Lloyd, 1990), and BOX elements (Martin et al., 1992). The REP elements are extragenic palindromic units with a variable loop in the proposed stem-loop structure. ERIC sequences are located in extragenic regions of the bacterial genome and contain a highly conserved central inverted repeat. BOX elements are located in intergenic regions and consist of differentially conserved modules, named boxA, boxB, and boxC, with boxB being the basic element around which the modular structure of BOX is organized. BOX elements can form stem-loop-structures due to their dyad symmetry. Primers for different representatives of these repetitive elements were established and proved useful in subtyping of many diverse Gram-negative and Gram-positive bacteria from multiple phyla (Olive and Bean, 1999; Versalovic et al., 1994). All three sets of repetitive elements could be identified in *P. larvae* allowing the use of various corresponding primers and primer pairs for subtyping (Alippi and Aguilar, 1998a,b; Alippi et al., 2004a,b; Antunez et al., 2007; Genersch and Otten, 2003; Loncaric et al., 2009; Peters et al., 2006). Comparing these studies reveals that reproducibility sometimes seems to be a problem with rep-PCR. Using BOX primers on Argentinean samples resulted in three or four banding patterns discriminated by a band of round 500 bp (Alippi and Aguilar, 1998a,b). Later, the discriminating band was described to migrate around 700 bp (Alippi et al., 2004b) and the patterns of the proposed genotypes A, B, C, and D were quite distinct from those shown in earlier studies indicating problems with intralaboratory reproducibility. On the other hand, the discriminating band of around 700 bp in the BOX pattern correlated with results obtained in different laboratories with independent collections of German (Genersch and Otten, 2003; Peters et al., 2006) and Austrian *P. larvae* isolates (Loncaric et al., 2009) suggesting that it is possible to optimize this method to achieve even interlaboratory reproducibility. The same is true for REP and ERIC primers. First, REP primers but explicitly not ERIC primers were described as being able to differentiate between the subspecies *P. l. larvae* and *P. l. pulvifaciens* (which at that time still existed) (Alippi and Aguilar, 1998b) whereas in a later study the differences in ERIC banding patterns between *P. l. larvae* and *P. l. pulvifaciens* provided the basis for an alleged subspecies specific PCR detection method (Alippi et al., 2004a). Differences in ERIC banding patterns correlating in part with the former subspecies classification within the species *P. larvae* could indeed be verified (Genersch et al., 2006).

To date, three BOX patterns are described in Europe (Genersch and Otten, 2003; Loncaric et al., 2009; Peters et al., 2006) and three to four on the American continent (Alippi et al., 2004b; Antunez et al., 2007); four REP patterns obtained with MBO REP1 primers (Versalovic et al., 1995) are described in Europe (Kilwinski et al.,

2004; Loncaric et al., 2009) and four REP patterns obtained with REP1R-I and REP2-I primers (Versalovic et al., 1991) were identified in a study including 323 strains from the American continent, 49 strains from Eurasia, nine strains from New Zealand, and one strain from Tunisia (Alippi et al., 2004b). Unfortunately, these results cannot be compared with the results from the European studies because different primers were used. Considering the global spread of AFB, it should be an aim for the future to develop a reliable international database for *P. larvae* based on a reproducible subtyping method applied by various laboratories to field isolates. Such a database will improve epidemiological studies of *P. larvae* tremendously.

5. Genotypes and virulence

The most important subtyping of *P. larvae* turned out to be the one obtained by using ERIC primers. Four ERIC genotypes, ERICI–IV, could be identified so far. According to the nomenclature used (Genersch et al., 2006) the former subspecies *P. l. larvae* comprises the genotypes ERIC I and ERIC II while the former subspecies *P. l. pulvifaciens* is now designated as *P. larvae* genotypes ERIC III and ERIC IV. Genotype ERIC I includes all strains which comply with the description of the former subspecies *larvae* according to Heyndrickx and co-workers (Heyndrickx et al., 1996), whereas genotype ERIC II, although causing AFB-outbreaks, was frequently misclassified due to its orange colony pigmentation considered characteristic for *P. l. pulvifaciens* only (Genersch et al., 2005, 2006; Heyndrickx et al., 1996). Genotypes ERIC III–IV meet most of the characteristics described for the former subspecies *pulvifaciens* (Heyndrickx et al., 1996). Epidemiological studies showed that ERIC I can be frequently isolated from foulbrood diseased colonies in Europe and on the American continent, whereas ERIC II seems to be restricted to Europe. ERIC III/IV has not been identified in field isolates in recent years so far and only a few strains exist in culture collections (Alippi et al., 2004b; Antunez et al., 2007; Genersch et al., 2006; Genersch and Otten, 2003; Loncaric et al., 2009; Peters et al., 2006). Therefore, *P. larvae* ERIC I and II are the two practically most important genotypes.

The four genotypes were shown to differ in phenotype including virulence (i.e. the measurable ability of a pathogen to cause disease equaling the disease producing power of a pathogen (Shapiro-Ilan et al., 2005)) at the larval stage (Genersch et al., 2005, 2006). Exposure bioassays revealed that members of ERIC II–IV are highly virulent against larvae in terms of the time course of mortality. All larvae infected with these genotypes are killed within approximately 7 days (Genersch et al., 2005, 2006). This means that only a minor proportion of the larvae die after cell capping resulting in the described clinical symptoms of AFB (ropy stage, foulbrood scale). In contrast, genotype ERIC I needed around 12 days to kill all infected larvae and, hence, is considered less virulent than ERIC II–IV for the individual larva (Genersch et al., 2005, 2006). Recently, it was shown that virulence at the larval stage is negatively correlated to virulence at the colony level due to the hygienic behavior of the nurse bees (Rauch et al., 2009). It could be demonstrated that the removal rate of infected larvae depended on the time course of disease progression. Larvae infected by a representative of the fast killing (at the larval stage) genotype ERIC II were removed more efficiently (up to 90% removal rate) than larvae infected by a representative of the slower killing (at the larval stage) genotype ERIC I (around 60% removal rate). Removal of diseased larvae by nurse bees prior to the production of infectious spores efficiently disturbs disease transmission and disease development within this colony. The more AFB-infected larvae become moribund or even die before cell capping the more larvae will be removed by nurse bees as part of the social immune defense (Spivak and Reuter, 2001; Wilson-Rich et al., 2009; Evans and Spivak, 2010), the less ropy mass and spore containing foulbrood scales will be produced, and, consequently, the more slowly the disease will develop and spread within the colony. A low level of spore production may lead to slow spread within the colony which in turn may result in a slower collapse of the colony. In contrast, high level spore production may lead to fast within-colony spread and to a rather fast colony collapse. In other words, *P. larvae* virulence at the individual-insect level and colony level are negatively correlated (Rauch et al., 2009).

The non-pigmented *P. larvae* ERIC I can be considered the "classical" *P. larvae* genotype most probably causing the majority of AFB-outbreaks worldwide. So far, all type and reference strains of the former subspecies *P. l. larvae* of different culture collections belong to this genotype. AFB-outbreaks caused by the non-typical pigmented *P. larvae* genotype ERIC II are not a rare event at least in Germany (Genersch and Otten, 2003; Kilwinski et al., 2004) and Austria (Loncaric et al., 2009) which clearly shows that even a fulminate progression of AFB with most of the infected larvae being removed by nurse bees (Rauch et al., 2009) can lead to colony collapse and a pest-like disease process. However, since *P. larvae* ERIC II was often misdiagnosed as *P. l. pulvifaciens* due to its colony pigmentation and since its isolation is hampered by routine diagnostic methods (Forsgren et al., 2008), the exact prevalence of this genotype as causative agent of colony collapse is difficult to determine (for review see (Ashiralieva and Genersch, 2006; Genersch, 2008)).

Only two reports are available on the isolation of *P. larvae* genotypes ERIC III and IV (former subspecies *P. l. pulvifaciens*) from powdery scales (Gilliam and Dunham, 1978; Katznelson, 1950), and studies on the pathogenicity of this bacterium yielded conflicting results in the past (Hitchcock et al., 1979; Katznelson and Jamieson, 1951). However, although no AFB-outbreaks caused by *P. larvae* genotypes ERIC III and IV have been reported so far, the pathogenicity (i.e. the potential ability to cause disease (Shapiro-Ilan et al., 2005)) of these genotypes is no longer in question (Genersch et al., 2006).

6. Virulence and transmission

Pathogens, which typically rely on a host for reproduction and survival, need to be transmitted from host to host to ensure their survival in the host population. Therefore, it is in the interest of a pathogen to optimize its transmission success over the lifetime of the infection. During infection the pathogen reproduces within the host thereby reducing host fitness. The degree of host fitness reduction equals pathogen virulence (i.e. measurable ability to cause disease) and influences the chances of pathogen transmission. The trade-off model for the evolution of virulence in pathogens depending on a living host for transmission (Ebert, 1998; Ebert and Herre, 1996; Ewald, 1983), therefore, links pathogen transmission and pathogen virulence. According to this model pathogen virulence can evolve in response to alternative modes of pathogen transmission. Vertically transmitted pathogens, i.e. pathogens which are transmitted from one host generation (parent) to the next host generation (offspring), will develop less virulent forms with little impact on host fitness since host reproduction is vital for pathogen transmission. In contrast, horizontally transmitted pathogens, i.e. pathogens which are transmitted within the host population independent from host reproduction, can evolve more virulent forms with high negative impact on host fitness.

So far, AFB has been considered the paradigm of a highly virulent bee disease horizontally transmitted within and – more importantly – between colonies (Fries and Camazine, 2001). However, although the within-colony transmission of *P. larvae* is

exclusively horizontal, it is well-known from field observations that some infected colonies remained strong and never developed clinical symptoms, i.e. ropy mass in capped cells (Hansen and Brodsgaard, 1999), implicating the possibility of less virulent forms which may be transmitted vertically between colonies through reproductive swarming. Indeed, recent studies showed that AFB is not only horizontally transmitted between colonies, e.g. through diseased, weakened colonies being robbed out by other colonies (Lindström et al., 2008b), but also vertically through swarming of strong although infected colonies (Fries et al., 2006). The data on differences in virulence at the colony level between different *P. larvae* genotypes (Rauch et al., 2009) also suggest that at least for *P. larvae* isolates which have a low virulence at the colony level and, hence, little negative impact on colony fitness at the beginning of the disease process (like representatives of ERIC II allowing an efficient social immune response by killing the majority of larvae before cell capping) vertical transmission at the colony level through swarming is likely to occur. One question, however, is still open in this context: Is swarming really a vertical transmission route for *P. larvae* on colony level or is it a cure, a way for the swarm (old queen with accompanying worker bees but without brood) to be cured from the disease? For successful vertical transmission, swarms should carry spores which should remain within the colony and infect new larvae to produce more spores to ensure the survival of the pathogen in the bee population or the environment. In contrast, for an effective cure, the swarm should be able to get rid of the spores by e.g. grooming before the next brood gets infected thereby disrupting disease progression and pathogen transmission. The question of vertical transmission of AFB was investigated by Fries and co-workers (Fries et al., 2006). They determined the level of *P. larvae* spores in swarms originating from both clinically diseased colonies (i.e. ropy mass in capped cells found upon inspection) and infected colonies without visible clinical symptoms. They demonstrated that in swarms from infected although not yet obviously diseased colonies, which were characterized in their study by less than 20 bacterial colony forming units per bee, the detectable spore level decreased drastically within 2 months post swarming and remained nearly undetectable for over 13 months indicating that no larvae were successfully infected in these colonies and, hence, no new spores were produced in these colonies. This is consistent with a study on the shook swarm method as an alternative way to control AFB demonstrating that artificial swarming is an effective way to cure part of the colony (the swarm) from AFB rather then transmitting the disease (Pernal et al., 2008). In contrast, in swarms originating from clinically diseased colonies, which had a spore load of approximately 6.000 bacterial colony forming units per bee, spore load decreased within five weeks post swarming but then increased again to detectable levels after 13 weeks indicative of some clinical disease and new spore production in the colony. Therefore, swarming of clinically diseased colonies may present an efficient vertical transmission route whereas (artificial) swarming of non-clinically diseased colonies controls AFB.

7. AFB control

Surveying the AFB literature of the last decade revealed an interest in finding new strategies for the control of the disease. Since AFB is a notifiable disease in many countries, measures against and treatment of AFB are often regulated by law and include the destruction of clinically infected hives. In some countries, antibiotics are allowed for the treatment of infected colonies but in most European countries, the use of antibiotics in the treatment of bee diseases is banned. In those countries allowing antibiotics, a common strategy for the prevention and treatment of affected colonies is the use of oxytetracycline hydrochloride (OTC) or sulfathiazole. However, several problems are associated with the extended use of antibiotics: (i) Antibiotics are not effective against the infectious spores, hence, they only suppress clinical symptoms and mask the disease but cannot cure AFB; (ii) chemical residues can persist in honey affecting its quality and safety for human consumption (Lodesani and Costa, 2005; Martel et al., 2006); (iii) antibiotics when fed to larvae and adult bees may affect the vitality of the brood and the longevity of the bees (Peng et al., 1992); and (iv) OTC- and sulfathiazole-resistance in *P. larvae* has become widespread (Evans, 2003; Lodesani and Costa, 2005; Miyagi et al., 2000; Mussen, 2000; Piccini and Zunino, 2001) making new searches for alternative antibiotics already necessary (Alippi et al., 2005; Gallardo et al., 2004; Kochansky and Pettis, 2005; Kochansky et al., 2001; Peng et al., 1996; Williams et al., 1998). However, even with the development of new antibiotics active against *P. larvae* the general problem of emerging resistant *P. larvae* strains will remain a serious one.

Hence, there is an urge to develop alternative treatment strategies, which so far follow three different directions: (i) Breeding for honeybee genetic stock showing an increased individual or social immune response against AFB (Evans, 2004; Spivak and Reuter, 2001; Wedenig et al., 2003); (ii) biocontrol through antagonistic bacteria (Alippi and Reynaldi, 2006; Evans and Armstrong, 2005; Evans and Armstrong, 2006; Evans and Lopez, 2004; Olofsson and Vasquez, 2008); and (iii) treatment with natural antibacterial substances like essential oils of various plants (Eguaras et al., 2005; Fuselli et al., 2008a,b, 2006, 2007, 2009; Gende et al., 2008; Gende et al., 2009) or propolis (Antunez et al., 2008; Bastos et al., 2008).

The idea to obtain honeybees which are better able to fend off AFB through an increased social immune response through controlled breeding has already proved successful. Honey bee colonies, selected for hygienic behavior on the basis of a freeze-killed brood assay demonstrated resistance to AFB (Spivak and Reuter, 2001). In this assay the time is recorded for colonies to detect, uncap and remove dead brood from a defined comb section that had been cut from a frame within the brood nest, frozen at −20 °C for 24 h, and placed in the nest of a test colony (Spivak and Reuter, 1998). These results for resistance to AFB were consistent with early studies which already demonstrated that the most important mechanism of resistance to AFB is the hygienic behavior of adult bees toward infected larvae (Rothenbuhler, 1964a; Rothenbuhler, 1964b). The usefulness of such breeding attempts are further supported through the recent observation that the hygienic behavior of nurse bees, expressed as detection and removal of diseased brood from the nest, has a clear impact on spore production and, hence, on disease transmission and disease progression within the infected colony (Rauch et al., 2009).

Biocontrol through antagonistic bacteria is a tempting idea and the first successful experiments with cultivated *P. larvae* have been presented (Evans and Armstrong, 2005; Evans and Armstrong, 2006). However, one characteristic of *P. larvae* is that it is consistently isolated as pure culture from AFB dead larvae (White, 1906) indicating that *P. larvae* itself obviously produces potent antibiotics as confirmed recently (Fünfhaus et al., 2009). Therefore, the efficacy of antagonistic bacteria against *P. larvae* infection *in vivo* remains to be shown.

Essential oils effective against *P. larvae* are also a promising idea especially since a combined treatment of the mite, *V. destructor* and *P. larvae* could be possible. But the efficacy of such a treatment and the absence of adverse side effects on bee vitality (Ebert et al., 2007; Higes et al., 1997) and honey quality still need to be evaluated in practice.

8. AFB research entering the molecular era

Over the past decades *P. larvae* attracted much attention and progress was made in elucidating several aspects of the biology and the pathomechanisms of this important honeybee bacterial pathogen. However, although the identification and examination of all virulence genes of *P. larvae* is a prerequisite to understanding the pathogenicity mechanisms of this bacterium, molecular data on pathogenicity or virulence factors are still scarce. Recently, a draft genome sequence of *P. larvae* consisting of 646 contigs has been published together with a preliminary annotation (Qin et al., 2006). The average contig length is given with 6217 bp, the largest contig is 58,621 bp in length, and the total contig length is about 4 Mb. These sequence data represent a significant contribution toward understanding this important pathogen and are hopefully the first step into the molecular era of AFB research with more extensive genome annotation and functional analyzes to follow. The sequenced strain originated from an AFB diseased colony from the USA implicating that the published sequence belong to the *P. larvae* genotype ERIC I since ERIC II is currently restricted to Europe. Sequence data from the other three genotypes were generated through a comparative genome analysis using suppression subtractive hybridization (SSH) (Diatchenko et al., 1996) revealing considerable differences between the genotypes at the genome level (Ashiralieva et al., 2008; Fünfhaus et al., 2009; Fünfhaus and Genersch, 2008) as already suggested by pulsed-field gel electrophoresis (Genersch et al., 2006; Pentikäinen et al., 2009).

Most bacterial pathogens are characterized by a certain class of genomic islands, called pathogenicity islands (PAI) (Hacker et al., 1997). PAI occupy large genomic regions with a size of about 10 to 200 kb in the majority of cases. PAI carry one or more virulence genes and hence, PAI are present in the genomes of a pathogenic bacterium but absent from the genomes of non-pathogenic members of the same species or a closely related species. A characteristic feature of PAI is a G + C content (percentage of guanine and cytosine bases) differing from that of the core genome. This feature is often used to identify new PAI (Schmidt and Hensel, 2004). For *P. larvae*, neither whole genome sequencing (Qin et al., 2006) nor comparative genomics (Fünfhaus et al., 2009) so far revealed the existence of genomic regions characterized by a G + C content significantly differing from 42.0–43.3 mol% (renaturation method), the G + C content determined for the *P. larvae* genome (Heyndrickx et al., 1996) suggesting that *P. larvae* lacks PAI.

Although these are preliminary data since the available draft sequence does not allow a conclusive analysis, it is interesting to reflect on possible reasons for the potential absence of PAI in *P. larvae*. Most pathogens lacking PAI are extremely adapted to their host and lost the ability to replicate outside the host in natural environments. In contrast, pathogens harboring PAI have a more flexible lifestyle; they can live in different hosts or at different body sites of the same host or even outside the host (Schmidt and Hensel, 2004). Therefore, the acquisition of a PAI just provided these bacteria with yet another habitat, namely the host, to live in. However, *P. larvae* is an extremely specialized bacterium. Vegetative bacteria have never been found outside infected honeybee larvae and complex media are necessary to accomplish germination, cultivation, and sporulation in the laboratory (e.g. Lloyd, 1986; Nordström and Fries, 1995; Plagemann, 1985) indicating that *P. larvae* indeed is extremely adapted to its host as is rather typical for pathogens lacking PAI. Future studies will reveal presence or absence of PAI in *P. larvae*.

Recently, the first systematic screening for variations in genome content and the presence of virulence-associated genes in different *P. larvae* genotypes that could be linked with differences in virulence was reported. Using comparative genomics, the authors demonstrated the presence of giant gene clusters coding for polyketide/non-ribosomal peptide synthetases (PKS/NRPS) and a first putative toxin gene (Ashiralieva et al., 2008; Fünfhaus et al., 2009; Fünfhaus et al., 2008; Fünfhaus and Genersch, 2008). While toxins are rather usual for pathogens, giant gene clusters are not (Reva and Tümmler, 2008). PKS/NRPS are large multimodular enzymes non-ribosomally producing non-ribosomal peptides (NRP) or polyketides (PK) of broad structural diversity with antimicrobial, antifungal, or antiparasitic activities. PKS and NRPS are encoded in so-called giant gene clusters which range between 5 and more than 25 kb in length. The synthesis of such giant proteins is demanding in terms of energy, time and substrates and, therefore, the gain of fitness through the achieved end product, in this case antibiotics, must be substantial. Otherwise a bacterium would not synthesize such large enzyme complexes at its limit of translational ability and then consume further energy for the non-ribosomal synthesis of secondary metabolites. A possible gain of fitness for *P. larvae* through the production of antibiotics like PK and NRP is that these compounds comprise potent weaponry against competitors for the same niche, i.e. the larval midgut with its microflora presumably consisting of potential antagonistic bacteria (Evans and Armstrong, 2005; Evans and Armstrong, 2006). The success of *P. larvae* in colonizing the larval midgut and out competing the larval microflora has been demonstrated recently (Yue et al., 2008) and can also be deduced from the fact that saprophytes are not involved in degrading larval remains but instead pure cultures of *P. larvae* can be cultivated from AFB dead larvae (White, 1906). However, further studies are needed to elucidate the exact nature of these *P. larvae* antibiotics and the role these compounds play in the infectious process.

Several plasmids have been described in the past to occur in different strains of *P. larvae* (Benada et al., 1988; Bodorova-Urgosikova et al., 1992; Neuendorf et al., 2004) but none of those plasmids have been analyzed in more detail. Recently, a novel plasmid designated pMA67 was isolated from OTC-resistant strains of *P. larvae* and it was demonstrated that this plasmid carried a tetracycline resistance gene (Murray and Aronstein, 2006) thus solving the long standing question of the origin of tetracycline resistance in *P. larvae* (Evans, 2003). It was shown that the plasmid encoded *tetL* gene alone is sufficient to confer resistance to tetracyclines (Murray et al., 2007). Plasmid pMA67 encoded *tetL* is the first tetracycline resistance gene found in the genus *Paenibacillus* although PCR evidence exists suggesting that *Paenibacillus alvei* might also carry the same or a similar plasmid. Plasmid pMA67 is predicted to be mobilizable, which is a prerequisite for the exchange of the plasmid (or a close relative) between *P. larvae* and other bacteria like *P. alvei* sharing the same habitat (Murray et al., 2007).

9. Outlook

The aim of this review was to show how far we have come to understand *P. larvae* and the pathogenesis of this bacterial infection within the last century and how close we are to combating the disease. One important step had been the reclassification of the species *P. larvae*, which solved a long standing problem in AFB diagnosis and opened at last the possibility to unambiguously identify *P. larvae* in the lab via PCR-based detection methods. An improved or even optimized laboratory diagnostic is a key factor in successfully combating the disease. Epidemiological studies are another key factor. Sound epidemiological studies internationally cross-linked and following approved subtyping protocols are a prerequisite to better (i) recognize outbreaks of AFB, (ii) detect cross-transmission of *P. larvae*, (iii) determine the source of the infection and particular virulent strains, and (iv) monitor prevention and treatment programmes. Although a lot of effort has been

put into developing sustainable treatment strategies, little progress is evident. Treating infected hives even with new antibiotics will remain problematic; the idea of antagonistic bacteria combating *P. larvae* in infected larvae is still just at a preliminary experimental stage; essential oils are not yet proven to be really effective in the field without having adverse side effects on the bees; and an AFB tolerant bee stock which is as manageable as the 'normal' bees is not yet available, although it is presumably the best solution for the time being. Another key to the treatment of AFB is to better understand the complex host-pathogen interactions during infection. Knowing in detail down to the molecular level how successful infection is achieved by *P. larvae* should help develop innovative treatment regimes. Considerable progress has been made in unraveling the pathogenesis of *P. larvae*. We now know how *P. larvae* behaves in infected larvae in the early phases of infection but still most of the steps in the infectious process remain elusive. With the availability of the genomic sequence of *P. larvae* and methods to manipulate the bacterium (Murray and Aronstein, 2008) unraveling these processes at a molecular level will now be feasible. However, a lot of work still needs to be done before we will understand the complex interactions between *P. larvae* and its host, the honeybee larva and even more work is necessary before we will be able to develop a cure for AFB based on these results.

Conflicts of interest

There are no conflicts of interest to be declared.

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